Optimizing Cellular Attachment and Function in Long-Term Hepatocyte Cultures Using Polyelectrolyte Multilayer Surface Modification

Jonathan Wu

SUBMITTED TO THE DEPARTMENT OF MECHANICAL ENGINEERING IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

JUNE 2006

Signature of Author: _____
Jonathan Wu
Department of Mechanical Engineering
June 2006

Certified by: _____
Linda G. Griffith
Professor of Mechanical Engineering
Professor of Biological Engineering
Thesis Supervisor

Accepted by: _____
John H. Lienhard V
Professor of Mechanical Engineering
Chairman, Undergraduate Thesis Committee

Optimizing Cellular Attachment and Function in Long-Term Hepatocyte Cultures Using Polyelectrolyte Multilayer Surface Modification

by

Jonathan Wu

Submitted to the Department of Mechanical Engineering
On May 12, 2006 in Partial Fulfillment of the
Requirements for the Degree of Bachelor of Science

Abstract

Hepatocyte morphology is known to be closely linked to cellular functions. As a result, morphogenesis is extremely important to attain organ-equivalent levels of tissue function from *in vitro* cultures. Thus, a challenge exists in designing materials suitable for supporting liver-derived cells that are not only biochemically hepatospecific but also biophysically sensitive to the mechanical nature of hepatocytes to achieve highly differentiated cell phenotype found in a natural liver. We employ a unique substrate material system of polyelectrolyte multilayers (PEM) that can be tuned to achieve mechanical compliances of several orders of magnitudes ($E_s = 10^5$ to $E_s = 10^8$ Pa). We have shown that PEM modification can effectively change the surface mechanical compliance, and, thus, hepatocyte morphology and attachment, by looking at varying PEM pH deposition conditions (pH 2.0, 4.0, and 6.5) and collagen concentrations (0, 3, 10 ug/cm^2) on different materials (tissue-culture polystyrene, polycarbonate, and Permanox). For all materials, PAH/PAA 4.0/4.0 provided the balance of cellular attachment that appeared neither confluent nor sparse while also promoting a natural hepatocyte phenotype. We also observed that PEM films can effectively mask any inherent substrate material properties. Therefore, the use of PEM modification can be applied to a variety of surfaces and geometries for hepatocyte cultures. We believe that PEM is an invaluable tool in optimizing cellular attachment and function and will prove to be essential to future *in vitro* hepatocyte studies.

Thesis Supervisor: Linda G. Griffith
Title: Professor Mechanical Engineering and Biological Engineering

Table of Contents

I. Introduction

Hepatocyte morphology is known to be closely linked to the functional output of the cells (Semler and Moghe, 2001; Singhvi et al., 1994a). Standard cell cultures that have been used in the past can successfully passage these hepatocytes; however, in certain instances hepatocellular functions become compromised because the cell no longer resembles a natural hepatocyte from a living liver. In many cases, specific cellular phenotypes are directly related to the cellular functions including cell survival, proliferation, differentiation, motility, and gene expression (Huang et al., 1998; Singhvi et al., 1994b). The morphogenesis and assembly have been well established to be pertinent in the functional performance of liver-derived cells *in vitro* (Hansen et al., 1998; Singhvi et al., 1994a; Torok et al., 2001; Yuasa et al., 1993). Thus, a need for a suitable substrate that can simulate, not only the biological, but the chemical and physical *in vivo* environment of a liver is of utmost importance to further *in vitro* hepatocyte experiments.

A major challenge exists in designing materials suitable for supporting liver-derived cell cultures. To attain organ-equivalent levels of tissue function from cultures, the growth substrates must not only be biochemically hepatospecific but also biophysically sensitive to its mechanical nature to achieve a highly differentiated cell phenotype (Semler et al., 2004). Physical resistivity of the cellular environment is thought to determine cell shape and, as a result, remodel the internal architecture of intracellular signaling processes (Davis et al., 2002; Huang and Ingber, 2000). One of the biophysical parameters thought to be intimately coupled with the outcome of hepatocellular morphogenesis is the substrate mechanical compliance because of the high sensitivity of a hepatocyte to the mechanics of the environment.

To date, there have been few approaches in studies that have used these methods of manipulating substrate mechanical compliance including the utilization of differentially compliant basement membrane hydrogel (Matrigel) substrates (Semler EJ et al. 2004). However, another method, in which we employ in our experiment, utilizes a unique substrate material system, weak polyelectrolyte multilayers (PEM). It has been shown that the PEM mechanical properties can be controlled directly through modulation of the component solution pH during PEM assembly relatively easily (Thompson et al. 2005). Using a layer-by-layer method of assembly of polyanions and polycations creates naturally formed ionic crosslinks which range a spectrum of compliance levels simply by targeting the pH to a certain value at which the films are assembled.

With the ability to directly control the substrate mechanical compliance of the PEM, we are able to quickly survey a range of biochemical and biophysical conditions to determine the most suitable system that best emulates an *in vivo* environment. Therefore, our ultimate goal is to investigate the use of polyelectrolyte multilayer (PEM) surface modification in optimizing cellular attachment and function in long-term hepatocyte cultures.

II. Background

Using weak polyions, like PAH and PAA, allows the creation of a wide variety of multilayer structures simply by adjusting the pH-sensitive charge density of the assembling polymers. PAA (pK_a ~ 5) and PAH (pK_a ~ 9) contain ionizable carboxylic acids and amines, respectively. Therefore, by targeting the pH during deposition, the degree of ionization of these weak polyelectrolytes (i.e., the number of COO^- vs COOH groups for PAA and the relative number of NH_3^+ vs NH_2 groups for PAH) can be tuned. Thus, the number of ionic bonds formed between the COO^- and NH_3^+ groups can be coordinated as well. The surfaces can therefore be coated with alternating layers of PAA and PAH adjusted to the same pH, resulting in ionically cross-linked PEMs.

In our experiments we will be using three different pH deposition conditions at pH 2.0, 4.0, and 6.5. When PAH and PAA are assembled at pH 6.5, both polymers are essentially fully charged molecules and, therefore, form thin ionically cross-linked layers. As a result, the PEM films are homogenously well-mixed, regardless of the terminal layer (Figure 1B). PEMs assembled at 2.0 are enriched by PAA chains both within and on the surface of the film. Therefore, these loopy PAH/PAA multilayers demonstrate little ionic cross-linking because most of the PAA groups exist in their uncharged, protonated COOH state (Figure 1A). PEM samples are typically described in literature by the cation/anion pair and assembly pH for each polyelectrolyte, for example, PAA/PAH 6.5/6.5.

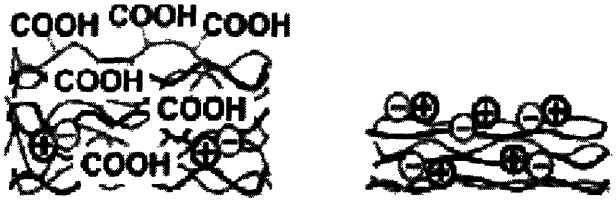


Figure 1: Schematics of the (A) 2.0/2.0 and (B) 6.5/6.5 PAH/PAA multilayer assemblies shown with PAA as the terminal layer. (Mendelsohn et al. 2003)

In a previous study by MT Thompson et al (2005), nanoindentation of fully hydrated PAA/PAH multilayers were conducted to quantify the effect of multilayer assembly pH on mechanical compliance of the PEMs in terms of E_s, the Young's modulus for the substrate material. Figure 2A shows that E_s varies significantly with the assembly pH, but does not vary to a statistically significant extent as a function of the terminal layer (PAA or PAH). Using PEMs, a broad spectrum of $E_s = 10^5$ to $E_s = 10^8$ Pa (an order of magnitude lower than TCPS) can be achieved. The terminal layer of the PEM is indicated as polyanion PAA (solid black) or polycation PAH (solid gray).

Although the terminal layer did not effect the overall compliance of the PEM, the film showed that post-seeding cell density increased as the compliance of the multilayer decreased. PAA-terminated PEMs showed greater cell attachment over a course of a 7-day observation than PAH-terminated PEMs for pH >2.0 (Figure 2B). Therefore, to explore the biophysical nature of hepatocytes, our experiments entailed examining the range of compliances using assembling pH 2.0, 4.0, and 6.5, but only using PAA terminal layers since it exhibited a larger cell count.

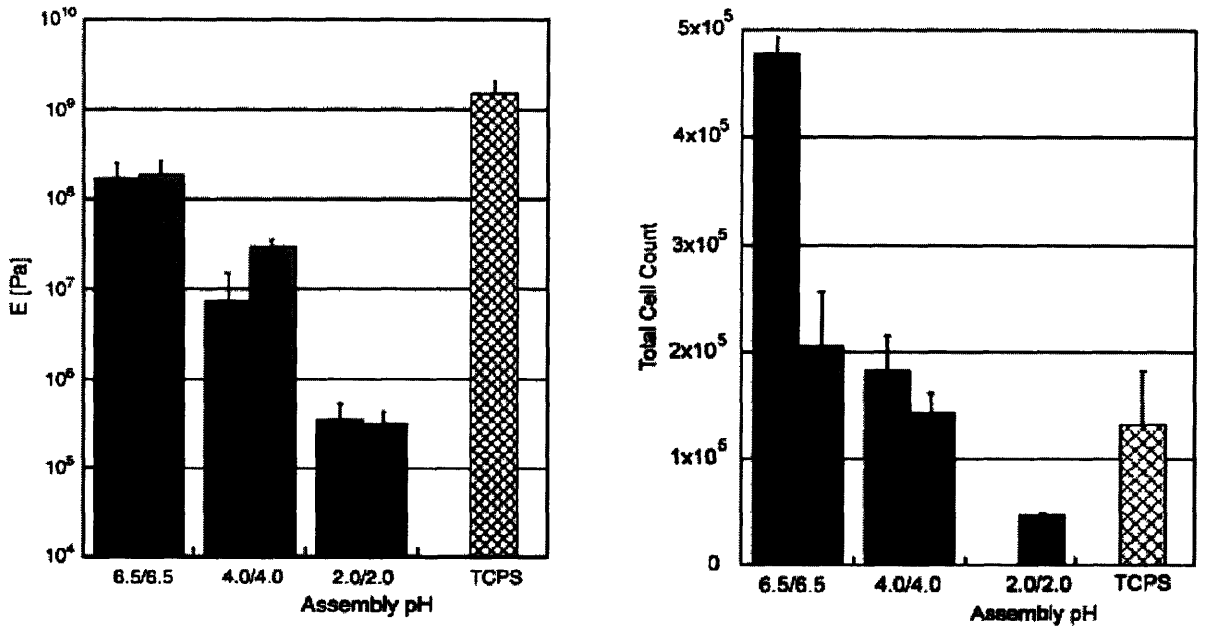


Figure 2: (A)Young's Modulus E_s as a function of assembly pH of PAH/PAA solutions, pH = 6.5, 4.0, 2.0. The terminal layer is indicated as PAA (solid black) or PAH (solid gray). (B)Total number of fibroblast cells harvested from 60 mm-diameter Petri dish at seven days post-seeding, as a function of PEM assembly pH. (Thompson et al. 2005)

In regards to the biochemical hepatospecifics, our experiments examined the use of Rat Tail Collagen Type I, a fibrous protein found in most tissues and organs used in *in vitro* cultures as a thin layer to promote cell attachment. Collagen I has been used in the maintenance of hepatocyte function, state of differentiation, and elevated levels of liver cell gene transcription (Sidhu JS et al., 1993; Li AP et al., 1992). Therefore, to explore the biochemical nature of hepatocytes on attachment and morphology, our experiments examined the range of collagen adsorption densities of 0, 3, and 10 ug/cm^2.

III. Collagen Adsorption/Removal Assay

To facilitate the growth of hepatocytes, collagen was initially adsorbed before cells were seeded on the surface, regardless of whether the substrate was modified with PEMs or not. However, it is not clear whether collagen adsorption is affected by PEM modifications. Therefore, our initial goal was to develop a protocol for quantifying our protein coating efficiency on polyelectrolyte multilayers. Initial trial experiments were done on untreated TCPS as a basis to develop a protocol to harvest adsorbed protein from a variety of surface substrates (TCPS, PEM-coated TCPS, PEM-coated PC, etc.) and surface geometries (plates, slides, various drilled scaffolds) and assay the amount of harvested protein as an indicator of protein coating efficiency.

Methods

Collagen Adsorption. Rat Tail Collagen (type I) solution (BD Biosciences) was used to coat the surfaces. The collagen adsorption protocol entailed coating duplicate wells with varying concentration of collagen diluted in PBS (0-400 ug collagen/ml) with a 12 hour incubation period at 37°C in a humidified incubator. A drying step followed for 2 hours in the laminar flow hood at room temperature. (For purposes of comparison, the standard Griffith Lab practice is to coat TCPS plates with ~30 ug/ml collagen (1:100 in PBS) for 2 hours at 37°C followed by 1-2 hours in the hood at room temperature. This condition corresponds to 3 ug/well in the figures below.) The supernatant was then separated from each sample into a different well to analyze the remaining collagen that did not adsorb.

Collagen Removal Solution. Two different concentrations of 1.0M and 0.5NaOH were used to harvest as a means to determine the basic strength on the harvesting efficiency. The wells were

treated for 15 minutes at the two basic concentration levels. The Pierce Micro BCA Protein Assay Kit was utilized to detect the amount of harvested protein from the plates. However, to minimize the interfering substance effects of NaOH, prior to the assay, the harvested samples were brought down to a workable pH(~7) range of the BCA assay which was accomplished by neutralizing the base with an equal molar volume of HCl.

BCA Protein Assay. The Micro BCA Protein Assay Kit (Pierce) was used as a colorimetric detection and quantization of total protein. 150ul of each sample was added to a 96-well plate along with 150ul of each collagen standard. To each well, 150ul of the assay working reagent was added. The plate was covered and then mixed on a plate shaker at 37°C for 1 hour. Following the incubation period, the absorbance of the plate was measured at room temperature at 562nm on a plate reader.

Results and Discussion

To provide appropriate standard curves for calculating collagen amounts using different harvesting solutions, two-fold serial dilutions of collagen were made in neutral solution (PBS), 1.0M NaOH/1.0M HCl, and 0.5 NaOH/0.5M HCl. NaOH and HCl are reported contaminants of the BCA assay at high concentrations. Figure 3 shows standards prepared and assayed on separate days (#1, #2). Collagen protein amounts were detected with decreased efficiency in the NaOH/HCl solutions. The assay showed limited linearity above ~6 ug/well. In calculating the amount of collagen harvested using NaOH (then neutralized using HCl), standard curves were used with corresponding solution molarity and standards concentrations >10 ug/well were neglected.

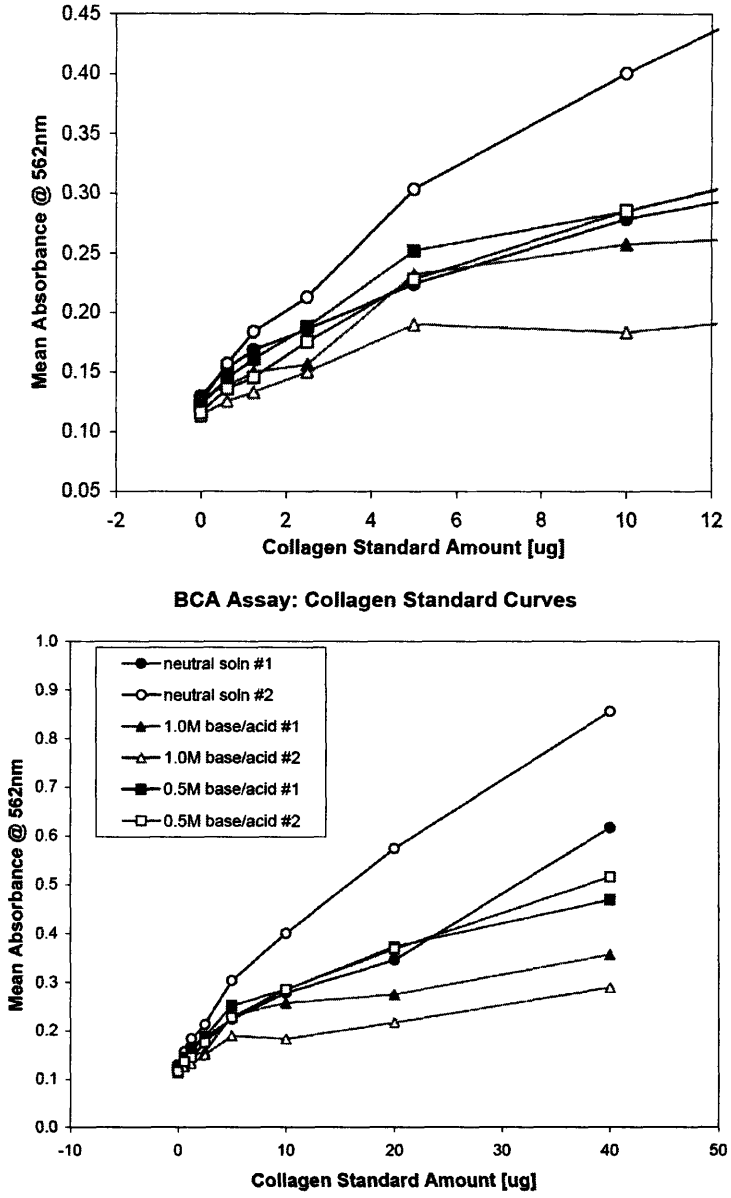


Figure 3: BCA Assay: Collagen Standard Curves

Figure 4 shows the mass balance of collagen adsorption on TCPS surfaces (using 96-well plates, with 0.35cm^2 SA/well). Plotted are the data points for the amount of protein that adsorbed to the surface at over a range of initial concentrations after the 12 hour incubation period (blue) and the amount of remaining protein found in the supernatant after the incubation period (green). Data is represented as the mean ± SEM of two independent experiments. The amount of protein

adsorbed to the surface and the amount remaining in the supernatant are roughly linear and rising with increasing adsorption solution concentration; however, there is a great deal of variability as seen in the error bars. The amount adsorbed to the TCPS surfaces showed substantial variability at high initial concentrations and possibly exhibited surface saturation at amounts >3 ug/well. The total protein detected (amount adsorbed + amount in supernatant solution) falls short of quantifying the amount of collagen and in some cases underestimates the amount by up to 25%. The amounts of protein reported were calculated using collagen standard concentrations in neutral (PBS) solution.

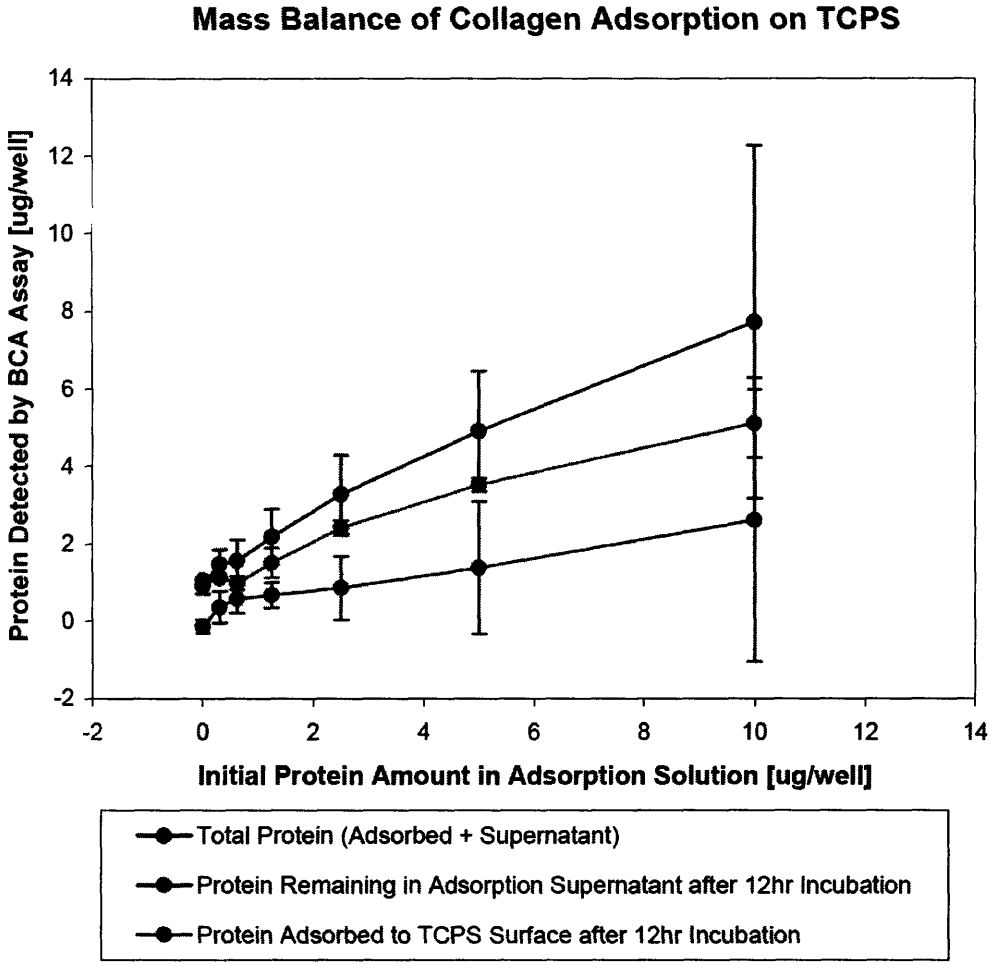


Figure 4: Mass Balance of Collagen Adsorption on TCPS

Figure 5 shows the comparison between the amount of protein adsorbed to the TCPS surfaces at the conclusion of the coating procedure and the amount detected after harvesting adsorbed protein using NaOH incubation. The amount of protein adsorbed was calculated for a range of initial coating concentrations (see Figure 4) using collagen standards in neutral solution. The amount of protein harvested was calculated using collagen standards in NaOH/HCl solutions of corresponding molarity. Data is represented as the mean ± SEM of two independent experiments.

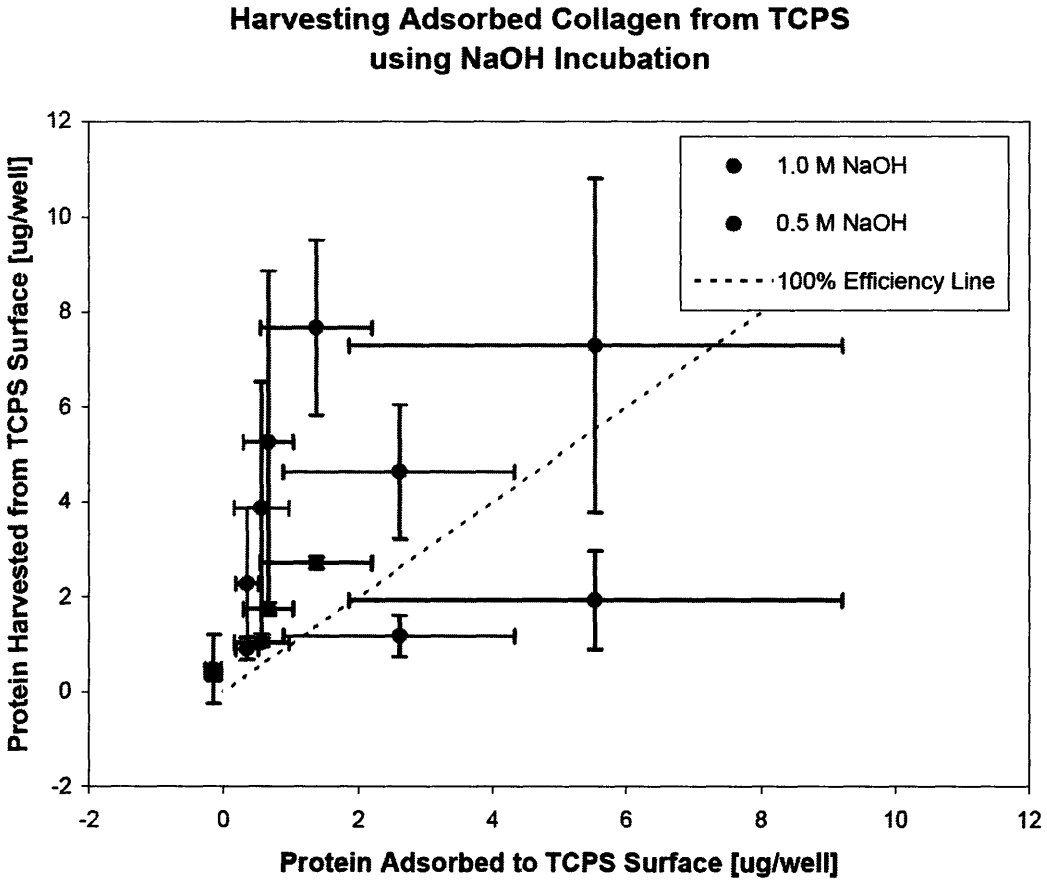


Figure 5: Harvesting Adsorbed Collagen from TCPS using NaOH Incubation

The protein harvesting using 1.0M NaOH incubation appears to be more efficient but is likely being over-estimated due to the high NaOH/HCl concentrations contaminating the BCA assay. The 0.5M NaOH harvesting seems to give more reasonable data and indicates that the harvesting procedure is fairly efficient up to ~2ug of adsorbed protein per well (which corresponds to ~10ug/well or 100ug/ml initial coating concentration). However, again, it is unlikely to be able to draw any conclusions from the data because of the extremely large error associated.

Conclusion

The Pierce Micro BCA assay does not appear to be an efficient method of determining collagen adsorption efficiency. Collagen is a hard protein to assay because of its non-remarkable side chains which make many available assay kits that depend on such markers unusable. Harvesting and quantifying adsorbed protein was carried out with both 1.0M NaOH and 0.5M NaOH incubations, and, although the 0.5M NaOH yielded less variable detection of collagen standards and what seemed more reasonable harvesting data than the 0.1M NaOH incubation, the data is inconclusive due to the inconsistency of results and the large calculated error. Thus, because our standard curves were not very sensitive and the data demonstrated even greater error from inconsistent results among duplicate trials, a different assay should be used to quantify collagen.

IV. Collagen Adsorption Efficiency on Untreated and PEM-treated Surfaces Experiment

Our goal was to determine the collagen adsorption efficiency of polycarbonate and Permanox, and to see the effects of collagen adsorption on different substrates and PEM conditions in comparison to tissue-culture polystyrene. The following variables were examined: polycarbonate and Permanox each with non-PEM-treated, pH6.5/6.5 PEM-treated, and pH2.0/2.0 PEM-treated surfaces; TCPS non-PEM-treated surfaces as a control; and all surfaces were coated with a collagen coating density of 0 ug/cm^2, 3 ug/cm^2, or 10 ug/cm^2.

Methods

Manufacturing Circular Substrates. The collagen adsorption efficiency of TCPS was tested using 24-well plates in the same manner as above. In order to test the adsorption efficiency of polycarbonate and Permanox, circular inserts with a surface area of 2.0cm^2 were machined from sheets of the material to fit at the bottom of 24-well plates. Only one trial of each condition was conducted due to limited PEM-treated surfaces at the time the experiment was performed.

Collagen Adsorption. The collagen adsorption protocol entailed coating wells at varying densities of 0 ug/cm^2, 3 ug/cm^2, and 10 ug/cm^2 with a shortened incubation period of 2 hours than described above at 37°C in a humidified incubator. A drying step followed for 2 hours in the laminar flow hood at room temperature. Because contamination of NaOH/HCl produced great variability among the assay results, only the supernatant left behind from the collagen incubation step was performed to analyze the remaining collagen that did not adsorb instead of the NaOH collagen removal method. The supernatant that was separated from each sample was transferred into a different 96-well.

BCA Protein Assay. The Micro BCA Protein Assay Kit (Pierce) was used as a colorimetric detection and quantification of total protein. 150ul of each sample was added to a 96-well plate along with 150ul of each collagen standard. To each well, 150ul of the assay working reagent was added. The plate was covered and then mixed on a plate shaker at 37°C for 1 hour. Following the incubation period, the absorbance of the plate was measured at room temperature at 562nm on a plate reader.

Results and Discussion

Figures 6A-F at the end of the section present the amount of collagen that remained in the supernatant of each condition. The graphs group the data of each material in two ways: by PEM surface modification and by initial collagen coating density. The data has been normalized in the respect that background noise (collagen level for "No PEM" and "0 ug/cm^2") for each individual material was subtracted from the rest of the data set since we were assuming that the value should be zero for non-PEM-treated surface with 0 ug/cm^2 initial coating density. All of the substrate conditions were done in single trials so no mean or standard deviations were obtained.

A rough collagen coating efficiency was determined by subtracting the theoretical amount of collagen added by the amount of collagen detected in the supernatant that did not adsorb. The polycarbonate data revealed that an unmodified surface has a higher collagen adsorption efficiency than that of the 6.5/6.5 or 2.0/2.0 modified surfaces (Table 1). This shows that for the case of polycarbonate, adsorption is effectively reduced with the modifications of PEM. Likewise, the Permanox data showed identical trends to the polycarbonate data. The unmodified Permanox also showed a greater adsorption efficiency over the 6.5/6.5 and 2.0/2.0 modified Permanox surfaces.

Both unmodified PC and Permanox surfaces had a slightly higher efficiency overall than TCPS comparatively. And in all cases, it shows from the rough efficiency calculations that the collagen adsorption efficiency was improved with the greater initial collagen coating density. This is opposite of what is expected. With the increase of collagen density, we would expect that the amount of collagen absorbed to saturate at a certain level and, thus, the coating efficiency to decrease as the initial coating concentration increases. It appears that the amount of collagen detected from the supernatant of both the PC and Permanox at 10 ug/cm^2 all peak around 50 ug/mL (~50% efficiency) which could be a direct consequence of the assay's poor inability to detect any higher concentrations of collagen.

	3 ug/cm^2	10 ug/cm^2	3 ug/cm^2	10 ug/cm^2	3 ug/cm^2	10 ug/cm^2
	71%	82%	78%	86%	73%	77%
	14%	44%	34%	51%		
	22%	42%	13%	34%		

Table 1: Collagen Adsorption Efficiencies for Polycarbonate, Permanox, and TCPS using 3ug/cm^2 and 10 ug/cm^2 for PEM pH depositions of pH 6.5, 2.0, and untreated calculated by subtracting the theoretical amount of collagen added by the amount of collagen that did not absorb (N=1 for all conditions). Data shows a wrong trend between increased collagen density and adsorption efficiency due to the inability of the assay to detect large concentrations of collagen.

Conclusions

It seems to show that PEM-modified surfaces have an effect on the collagen adsorption efficiency of the substrate. The modification of the surfaces brought the efficiency down from anywhere between 1/2 to 1/6 of the efficiency of the untreated surface. However, it is not possible to make any clear relationships between the different collagen concentrations because of the limited and poor detection of the BCA assay. As mentioned in the previous experiment, the BCA assay does not appear to be a suitable method in quantifying collagen because of its high variability at high collagen concentrations.

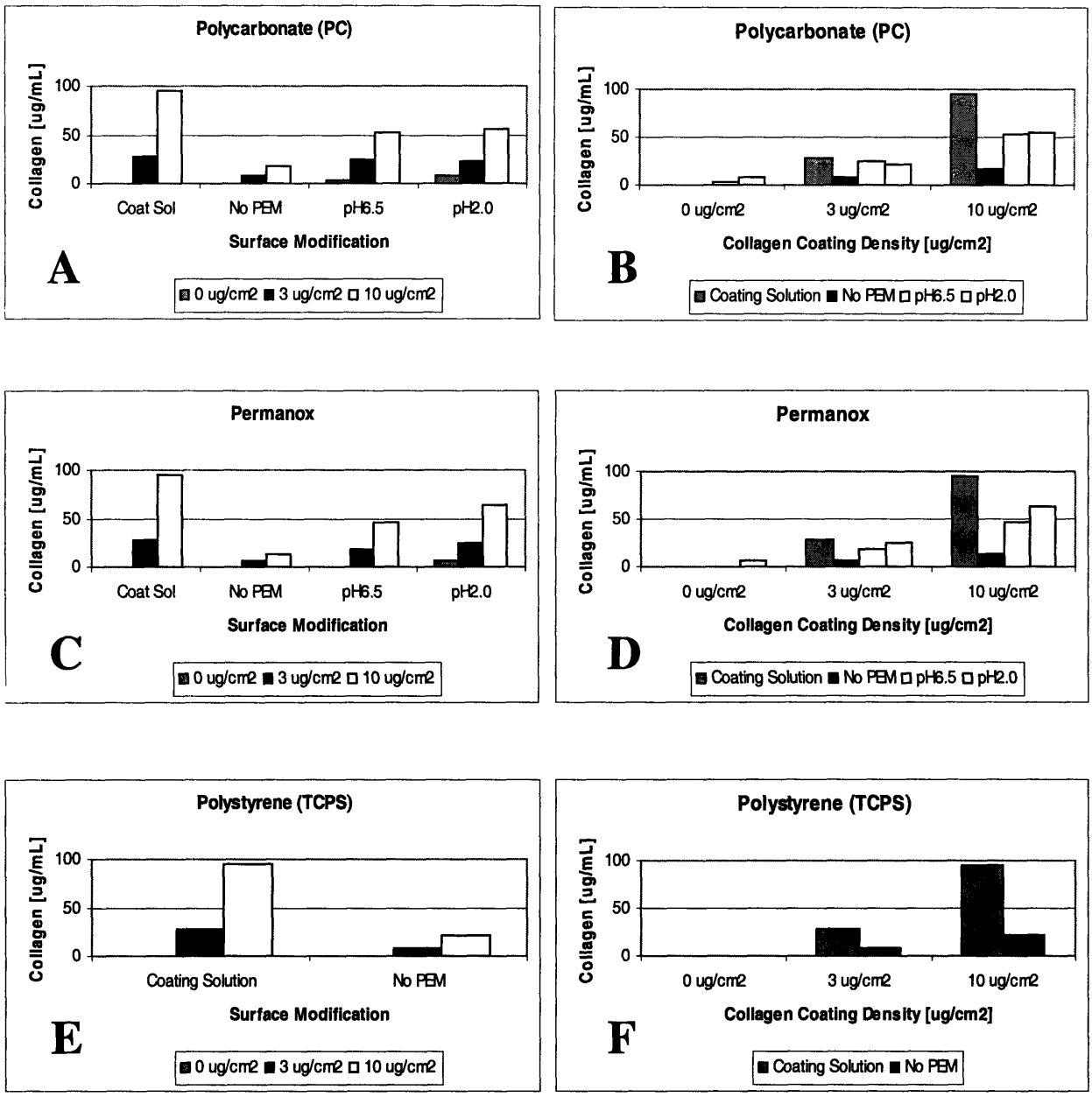


Figure 6: Graphs of amount of collagen remaining in supernatant after collagen incubation period that did not adsorb on the surfaces. Varying PEM compliances and collagen coating densities were explored (N=1 for all conditions) on (A) & (B) Polycarbonate (C) & (D) Permanox, and (E) & (F) TCPS. The first of the two graphs is sorted by the PEM surface modification and then followed by initial collagen coating densities.

V. PEM-Modified Hepatocyte Culture Experiment

Our main goal was to investigate the use of polyelectrolyte multilayer surface modification in optimizing cellular attachment and function in long-term hepatocyte cultures. As mentioned before, hepatocyte morphology is closely linked to its function output. Thus, our initial objective was to find an optimal condition where the proper amount of collagen and the optimal PEM deposition pH level would yield a more spherical morphology typically found in the liver.

Methods

Assembly of Weak Polymer Electrolyte Multilayers (PAH/PAA). Poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) were used to assemble PAA/PAH polyelectrolyte multilayers (PEMs). Dilute solutions (0.01M) of the two polyelectrolytes were prepared separately at a volume of 500mL with deionized water (Milli-Q). The solutions were stirred for 10 minutes to allow the polymers to dissolve. Upon mixture, the pH of each solution was adjusted to 2.0, 4.0, or 6.5 using HCl or NaOH. To avoid the precipitation of salt, ultimately affecting the charge chemistry of the final solution, only HCl or NaOH was used to prepare the solution to the target pH.

An automated dipping machine was used to coat polycarbonate and Permanox discs (surface area of 2.0 cm^2) and tissue-culture treated polystyrene using a custom manufactured apparatus machined from a CNC machine (Figure 7). All the surfaces were first sterilized with a five minute sonicating bath step and then with a 10 minute ethanol submersion. The substrates were first immersed in the polycationic solution (PAH) for 10 min followed by rinsing in three successive baths of deionized neutral water with light agitation for 2, 1, and 1 min, respectively.

The substrates were then immersed into the oppositely charged polyanionic solution (PAA) for 15 min and subjected to the same rinsing procedure. The process was repeated until the target number of layers was assembled. A PAA terminal layer (anion) was used for all of the samples in the experiments. The number of layers was varied to obtain a uniform dry (unhydrated thickness h = 40nm. For PAA/PAH PEMs assembled at pH = 2.0, there were 20 layers, at pH = 4.0 16 layers were used, and at pH 6.5 50 layers were used to coat the surface.

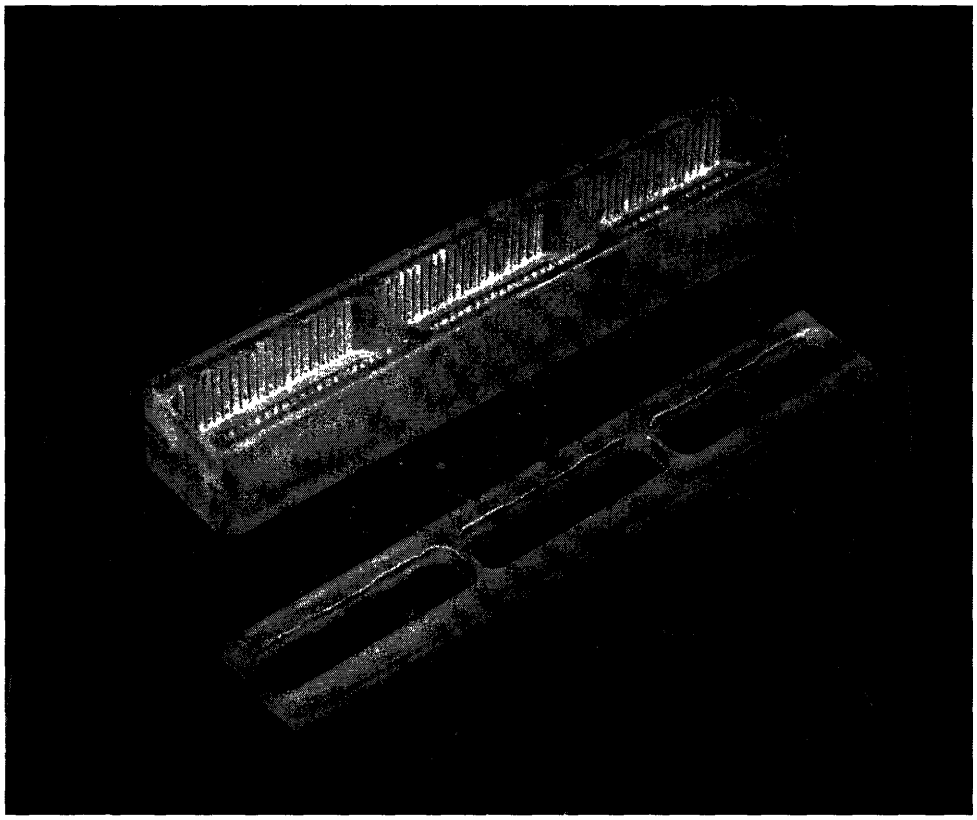


Figure 7: Custom manufactured apparatus from a CNC machine used for holding circular substrate discs during automated PEM deposition process.

Methylene Blue Staining. To qualitatively determine the efficiency of our staining, methylene blue, a small cationic dye which binds with free, charged carboxylic acids, was used to stain the PEM-treated surfaces. By dying the PAA/PAH films, the methylene blue binds to the COO^- groups that are not in coordination with NH_3^+ groups on the PAH chain. Because the conditions

under which the 6.5/6.5 modifications make almost all of the groups charged as each layer is deposited, the coordination between almost all of the charges and the film is tightly stitched together (high modulus). Thus, there were very few free carboxylic acid groups and not much binding of the dye occurred. Therefore, PEM 6.5/6.5 treated surfaces are the most clear. The 4.0/4.0 films have more free carboxylic acid groups. And as mentioned in the background, PEMs assembled at 2.0/2.0 are enriched with loopy PAA chains with carboxylic groups that cause little ionic cross-linking. Thus, 2.0/2.0 exhibited the greatest blue intensity.

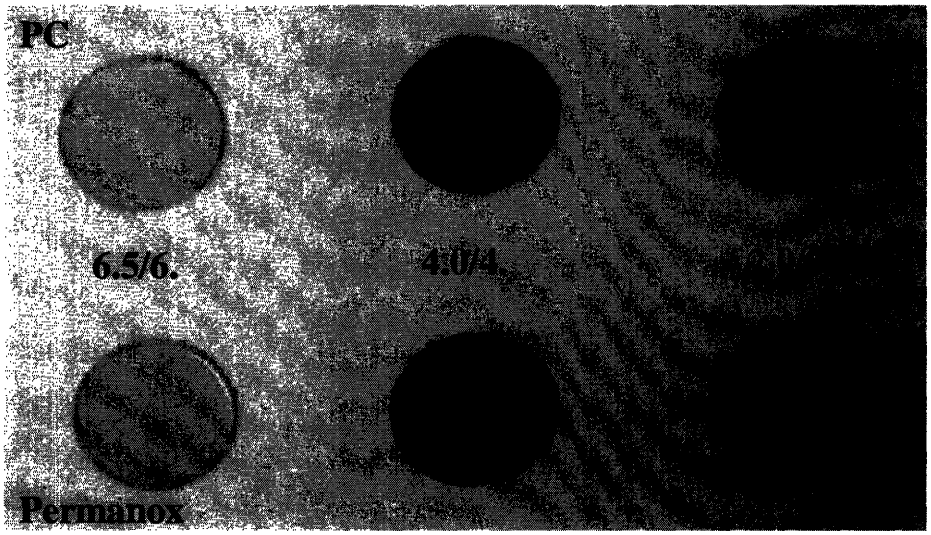


Figure 8: Methylene Blue Staining of PC and Permanox circular substrates. Methylene blue dye bound free carboxylic acids; therefore, because of the different degrees of ionic cross-linking from deposition pH, PAH/PAA 6.5/6.5 exhibited the clearest tint and PAH/PAA 2.0/2.0 exhibited the greatest blue intensity.

For our staining purposes, anhydrous methylene blue powder was dissolved with deionized water (Milli-Q) to 0.001 moles/L. The samples were submerged in the solution for 15 minutes and then rinsed twice with deionized water. Figure 8 shows the trends expected of the methylene blue stain. The 6.5/6.5 film is the most transparent and the 2.0/2.0 stain exhibits the darkest staining intensity for both the PC and Permanox sets, confirming a substantial amount of free acids both inside and on the surface. Between the two substrate sets, the staining intensities are

indistinguishable between each pH condition suggesting that material properties between substrates do not affect the appliance of the PEM films to the surfaces. The substrates were also compared to PEM-modified glass and TCPS substrates from the Rubner lab (not shown). Again, the staining intensities were nearly identical and varied as a result of PEM pH deposition, not material properties.

Collagen Deposition. Rat Tail Collagen (type I) solution (BD Biosciences) was used to coat the surfaces of the PEM-treated surfaces and the control surfaces. The collagen was dissolved 1:100 in PBS and added to a 24-well plate with 250ul collagen solution per well. The coated plates were then incubated for 2 hours at 37°C. At the end of the period the remaining solution was aspirated and the plate was left to dry in the hood for an additional 2 hours at 25°C.

Hepatocyte Isolation. Hepatocytes were isolated from male Fisher rats weighing 150-180 gm using a modification of the Seglen 2-step collagenase perfusion procedure (Powers et al. 2002). Cell yield and viability were determined via Trypan blue exclusion and hemocytometry. Typically, 250-300 million hepatocytes were harvested per rat liver with viability ranging from 85-92%. Following isolation, cells were initially suspended in Hepatocyte Growth Medium (HGM) and were subsequently diluted for culture seeding.

Hepatocyte Culturing. The cells were immediately transferred to the experimental and control surfaces. The surfaces were maintained at 37°C under 5%CO_2 in Hepatocyte Growth Medium (Block et al. 1996). Hepatocyte Growth Medium consisted of base medium DMEM (Gibco), supplmented with L-Proline, L-Ornithine, Niacinamide, D-(+)-Glucose, D-(+)-Galactose, Bovine

Serum Albumin, trace elements, Gentamycin (Sigma), L-Glutamine (Gibco), Insulin-Transferrin-Sodium Selenite (Roche), and Dexamethasone (Sigma). See Appendix for specifications.

Characterization of Cell Morphogenesis. Cellular morphogenesis was monitored at a magnification of 10x via transmitted light microscopy using a Zeiss Axiovert 100 microscope. At select time intervals after cell seeding, hepatocytes were viewed on PEM surfaces either during culture or after staining with Hoechst Nuclei stain. Several digitized images were acquired for each condition with a QImaging Retiga EXi Camera using the Improvision Openlab image processing software.

Results and Discussion

Figure 9A presents the results of the PEM-modified cultures on polycarbonate. The results are presented as a matrix of photos with the morphology and attachment of cells as a function of both PEM and collagen coating conditions. For the unmodified and uncoated PC substrate, the hepatocytes appeared elongated and sparse. As we expected, the addition of collagen facilitated better cell adhesion as seen on the substrates that were unmodified but coated with 3 and 10 ug/cm^2 of collagen. For the uncoated 6.5/6.5 substrate, very few cells attached as expected due to the absence of collagen, but, interestingly, the cells that did attach exhibited a more spherical morphology that is seen in living tissue. The addition of collagen to the 6.5/6.5 substrates produced significantly more cell attachment also with the same healthy spherical morphology. In the case of 4.0/4.0, the uncoated PC showed low cellular attachment but with the spherical morphology for those that did. The addition of collagen, again, showed a substantial increase in attachment. Likewise, 2.0/2.0 substrates demonstrated the same trends as both the 6.5/6.5 and

4.0/4.0 in terms of collagen coating and cellular attachment. In all cases, it was evident that PEM modifications effectively changed the morphology of the hepatocyte to a more spherically rounded state more commonly seen in the liver as opposed to a stretched and elongated morphology common in two-dimensional cultures. Also, there was an evident correlation between the PEM pH and the cellular attachment. The 6.5/6.5 (most rigid) showed significantly more adhesion than 2.0/2.0 as was also noticed by Mendelsohn et al. in their study of fibroblast cellular attachment.

Figure 9B displays the results of the PEM-modified cultures on Permanox. For the unmodified substrates, it was interesting to note the reduction in cellular attachment even with the addition of collagen compared to the polycarbonate substrates, perhaps due to the polymer chemistry of Permanox. Although the exact chemical structure of the commercial polymer is not readily available, the special, oxygen permeable properties may also have an effect on initial seeding of the cells. However, the addition of PEM seemed to mask the material properties of Permanox and, thus, the same trends apparent on PC were also seen for the PEM-modified Permanox substrates. Again, the non-collagen-coated substrates showed very little cellular adhesion and the cells that did bind on the PEM substrates displayed a spherical morphology. Also, the inverse correlation between mechanical compliance and cellular adhesion was also seen among the PEM substrates.

Figure 9C introduces the results of PEM-modified cultures on TCPS. The same general trends were apparent on the TCPS substrates. The unmodified and uncoated substrate showed the elongated morphology commonly seen on rigid surfaces. With the addition of collagen, the

hepatocytes became superconfluent with the stretched morphology. With the 2.0/2.0, we saw the other extreme with the most compliance. As a result, there was less cellular attachment and those that did adhere exhibited the rounder morphology. Interestingly unique to TCPS is the increased cellular attachment seen in comparison to PC and Permanox in the absence of collagen. This might be attributed to the chemistry of the tissue-culture treatment process of the polystyrene before it is marketed.

To obtain a better look at cellular attachment, a separate assay involved using live cell nuclei staining to better determine live cell count vs. external debris in assessing cellular attachment. Corresponding Hoechst stain cell images were taken using fluorescence microscopy for the PC and Permanox hepatocyte cultures (Figure 9D-G). As expected, the number of cells on the substrates without collagen was insignificant for all PEM conditions (Figure 9D). On the other hand, there was a substantially greater amount of cells with the collagen-coated substrates, as shown by the Hoechst stain images (Figure 9E). Again, the number of cells decreased with the more compliant 2.0/2.0 surface.

The non-collagen-coated Permanox substrates also showed a trivial amount of cells as to be expected (Figure 9F). The collagen-coated, PEM-modified substrates exhibited a greater amount of cellular attachment as confirmed by the corresponding Hoechst stain images (Figure 9G). However, the collagen-coated, unmodified substrate clearly demonstrated diminished attachment. As mentioned before, the chemistry of the Permanox polymer or the oxygen permeable properties may have a hand in the poorer binding affinity which, although, can be masked by the use of PEM.

Conclusion

PEM modifications can effectively change the surface mechanical compliance and, thus, the morphology of hepatocytes as demonstrated above. It is clear that hepatocytes are in fact intimately linked to its mechanical environment and, therefore, respond physically in its morphology. Also, for all surfaces the collagen proved absolutely necessary in the attachment of the hepatocytes. In combination with the collagen (either 3 ug/cm^2 or 10 ug/cm^2), the PAH/PAA 4.0/4.0 substrates, for both PC and Permanox, seemed to provide the best balance of cellular attachment that appeared neither confluent nor sparse. However, in some cases one might prefer a higher cell density and, thus, the 6.5/6.5 would be more appropriate with its less compliant surface; for a lower cell count the 2.0/2.0 would be more suitable with the most compliant surface. Interestingly to note, all of the PEM modified substrates for all materials exhibited the same trends in cellular attachment and behavior. Thus, the results from the PC, Permanox, and TCPS assays have shown that PEM films can effectively mask any inherent substrate material properties.

Figure 9A

Polycarbonate (PC)

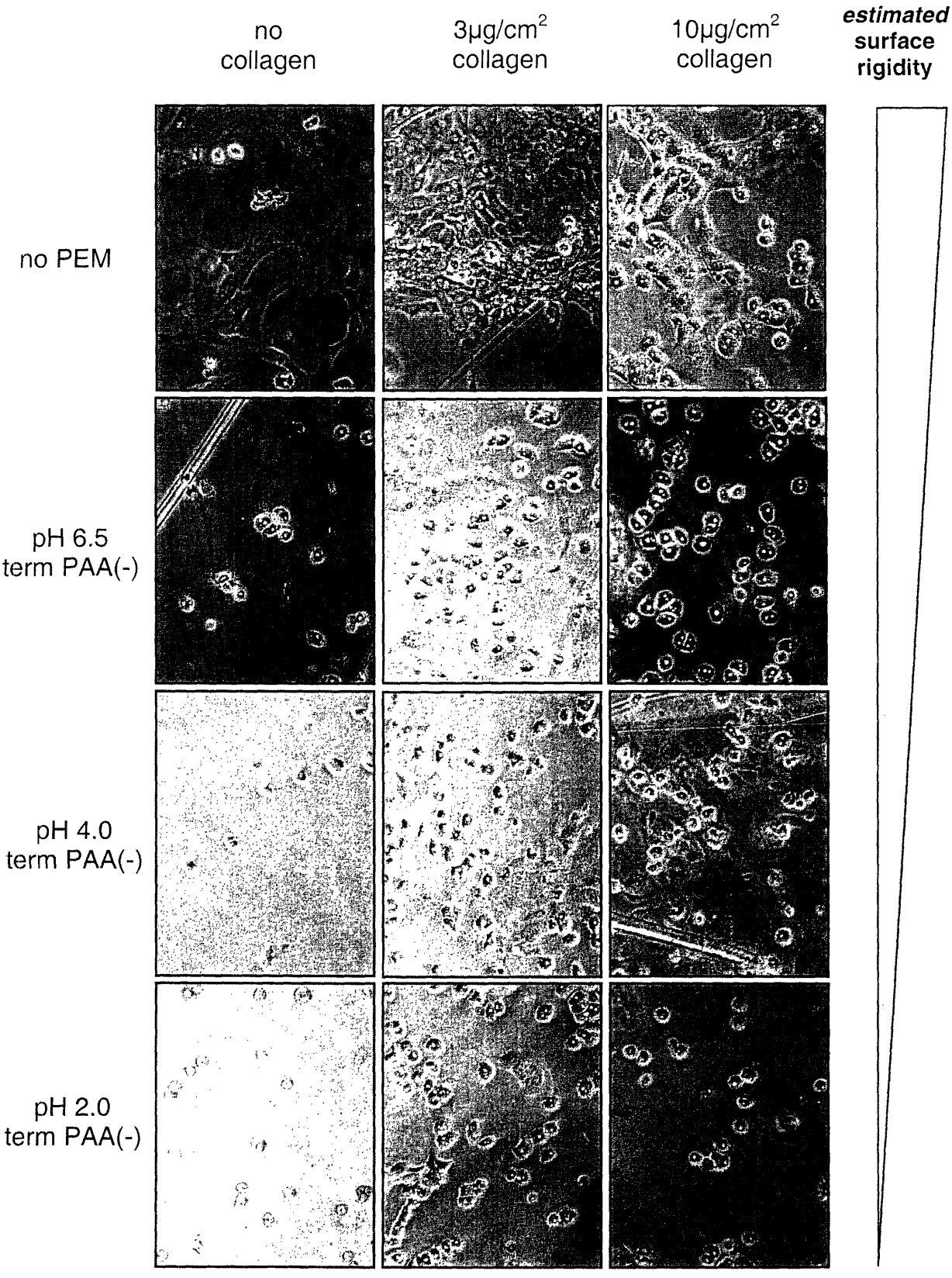


Figure 9B

Permanox

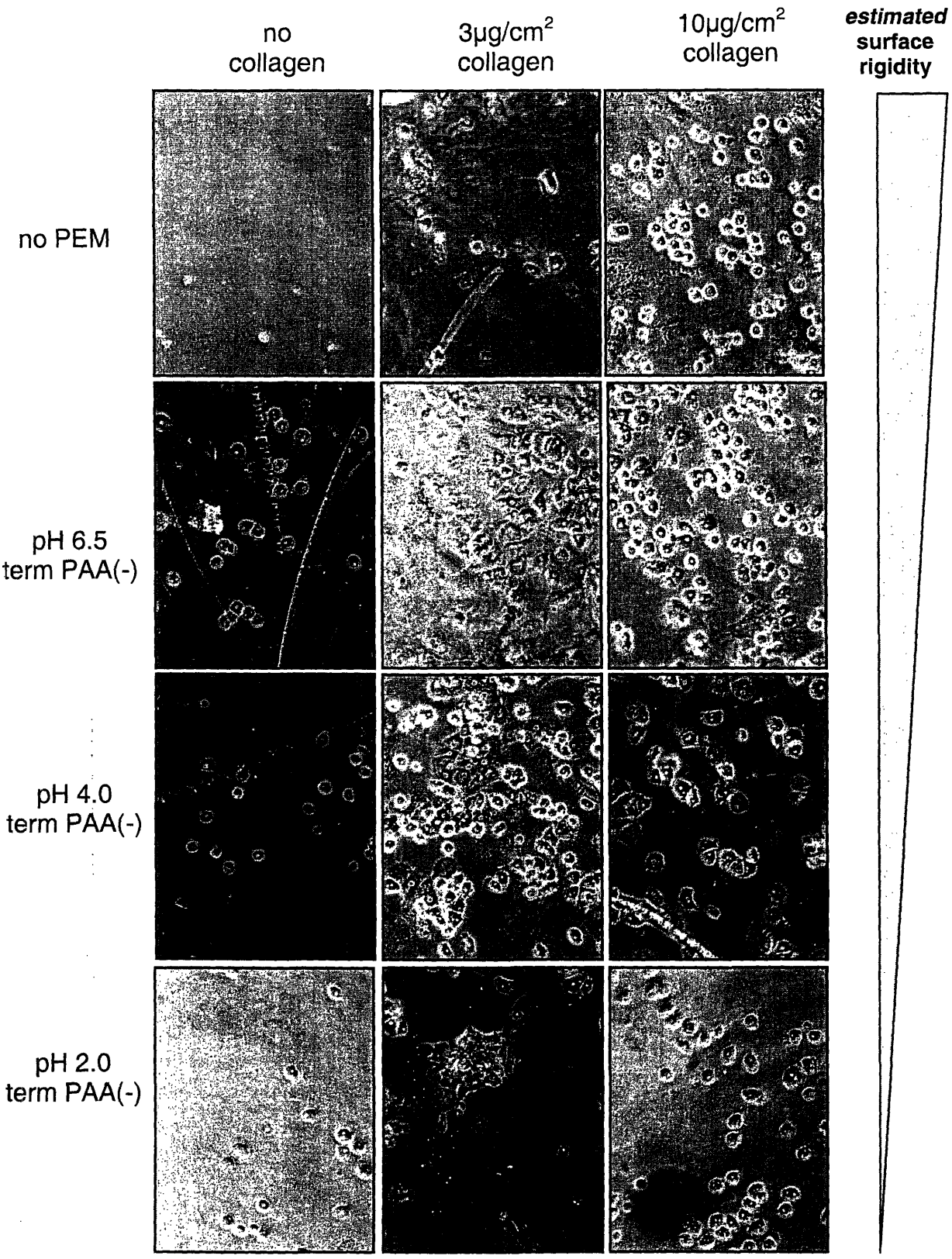


Figure 9C

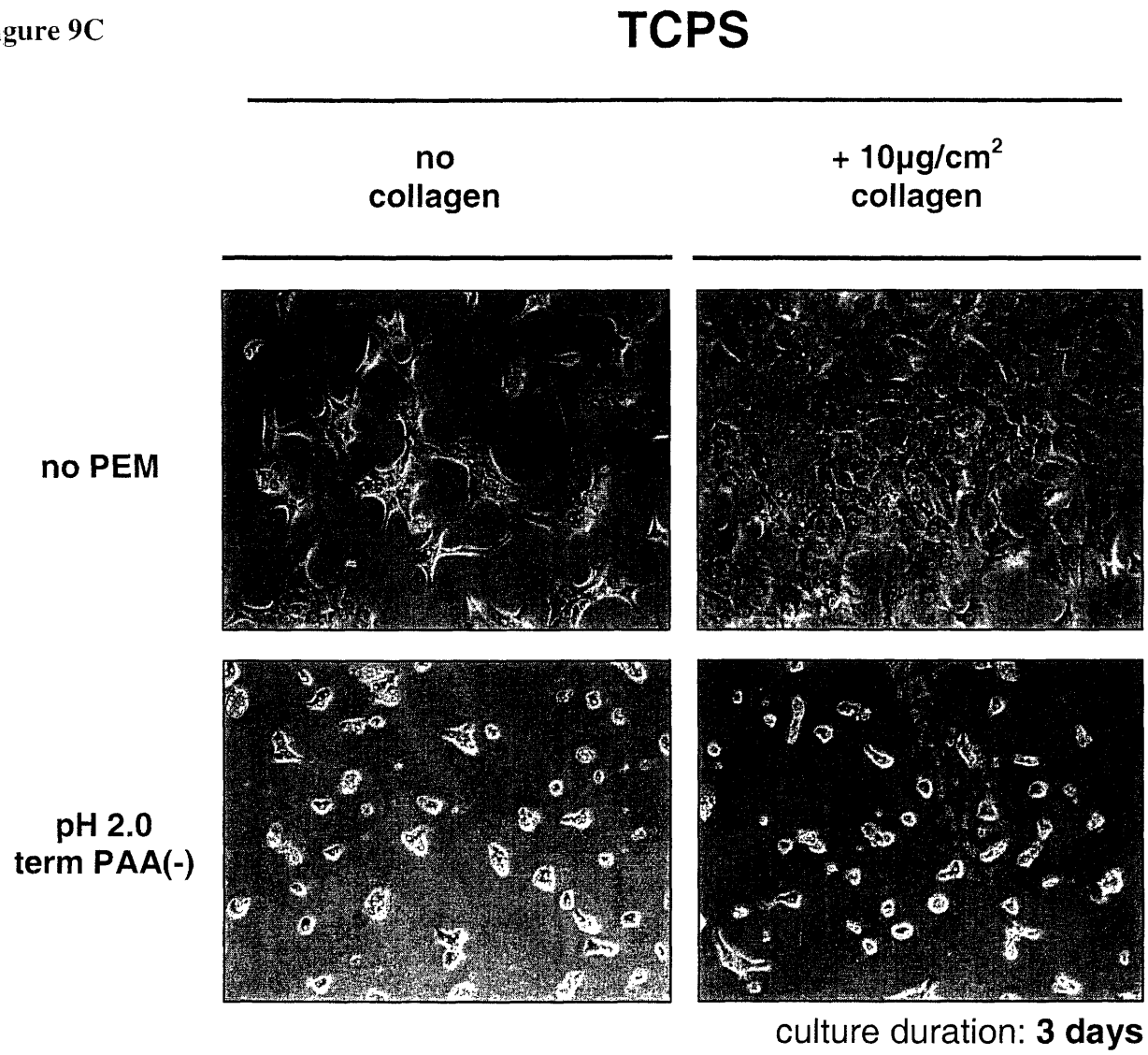

TCPS
no collagen
+ 10µg/cm2 collagen
no PEM
pH 2.0 term PAA(-)
culture duration: 3 days

Figure 9D

Polycarbonate (PC)

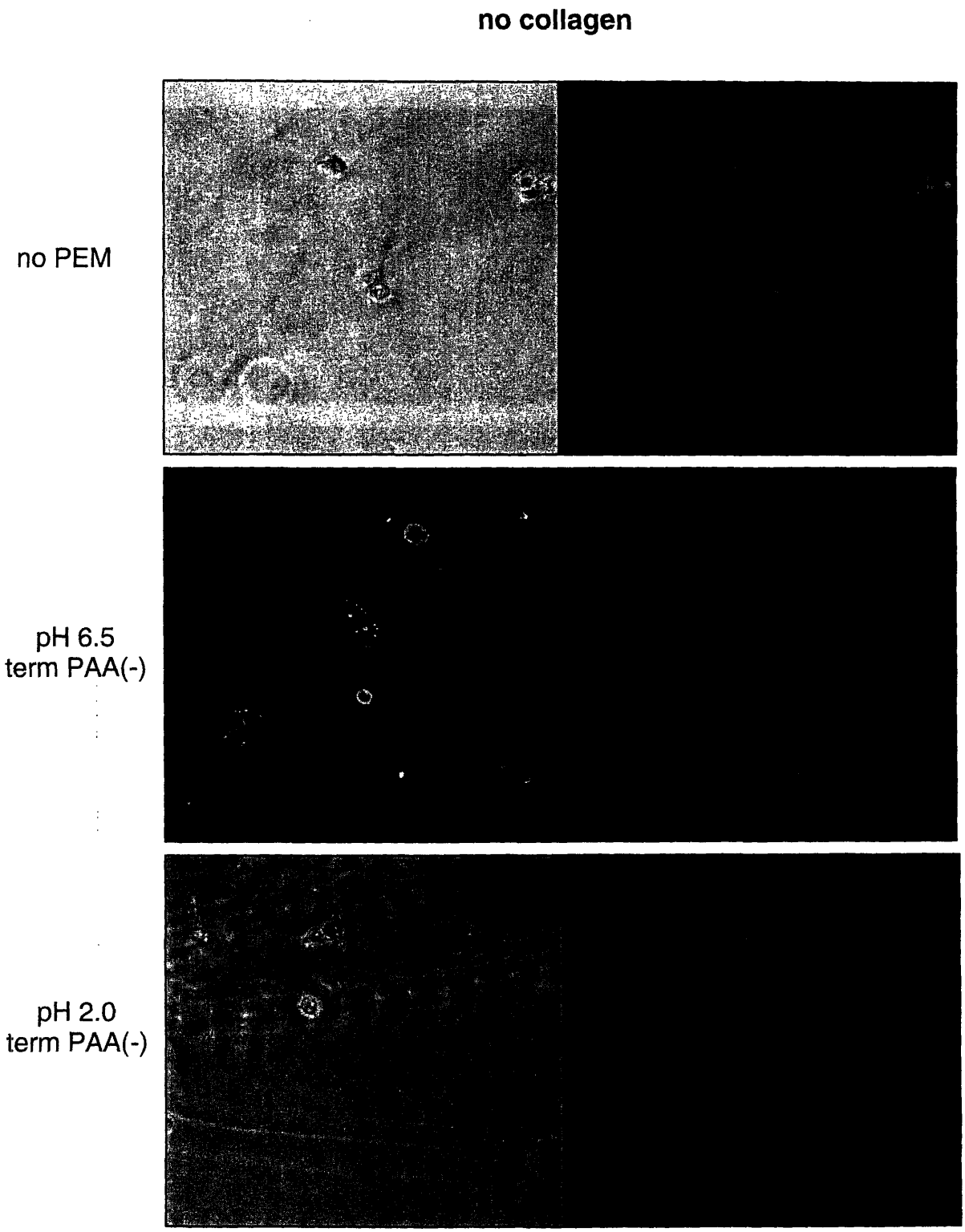
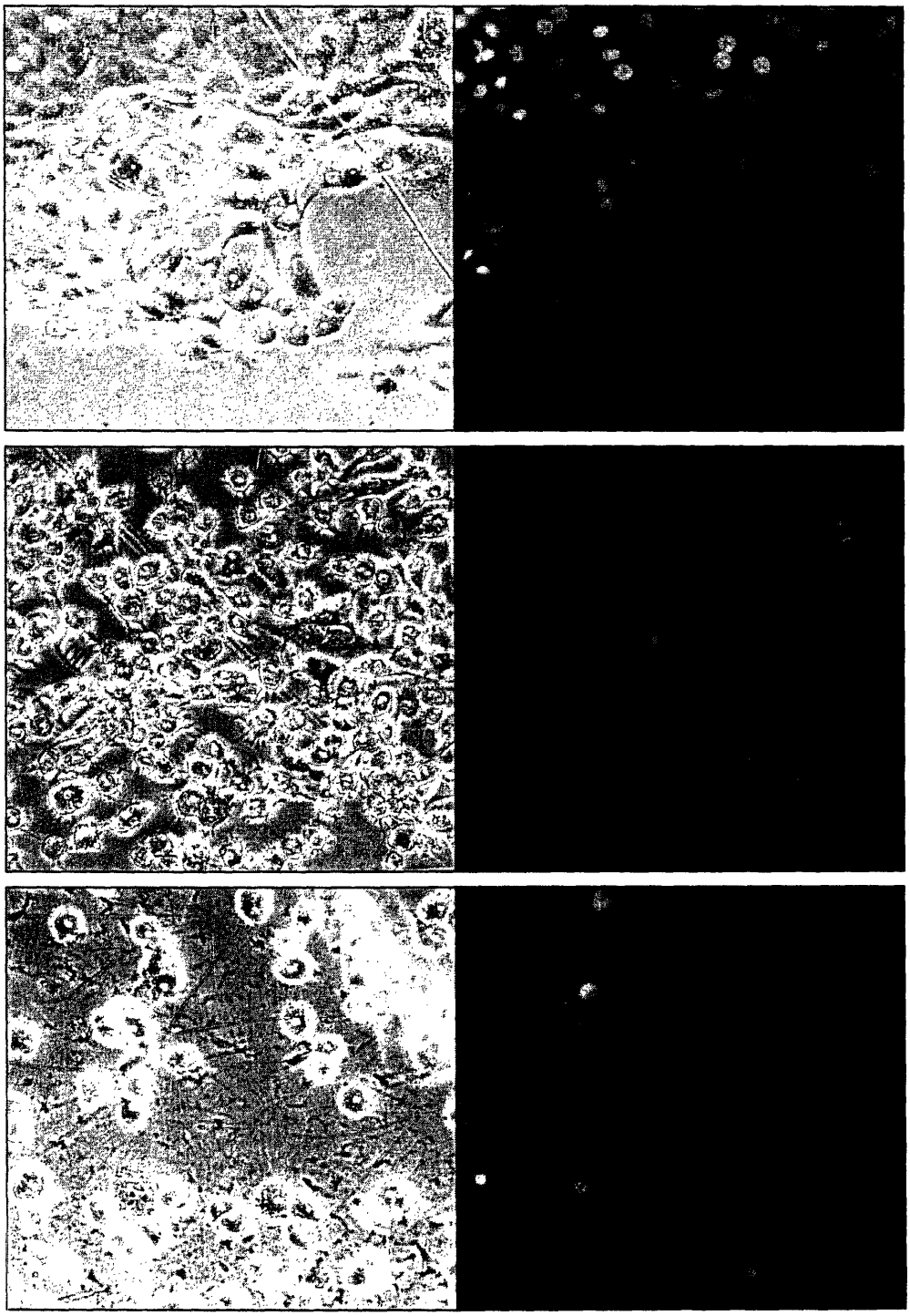


Figure 9E

Polycarbonate (PC)

+ 10μg/cm^2 collagen



culture duration: **4 days**

Figure 9F

Permanox

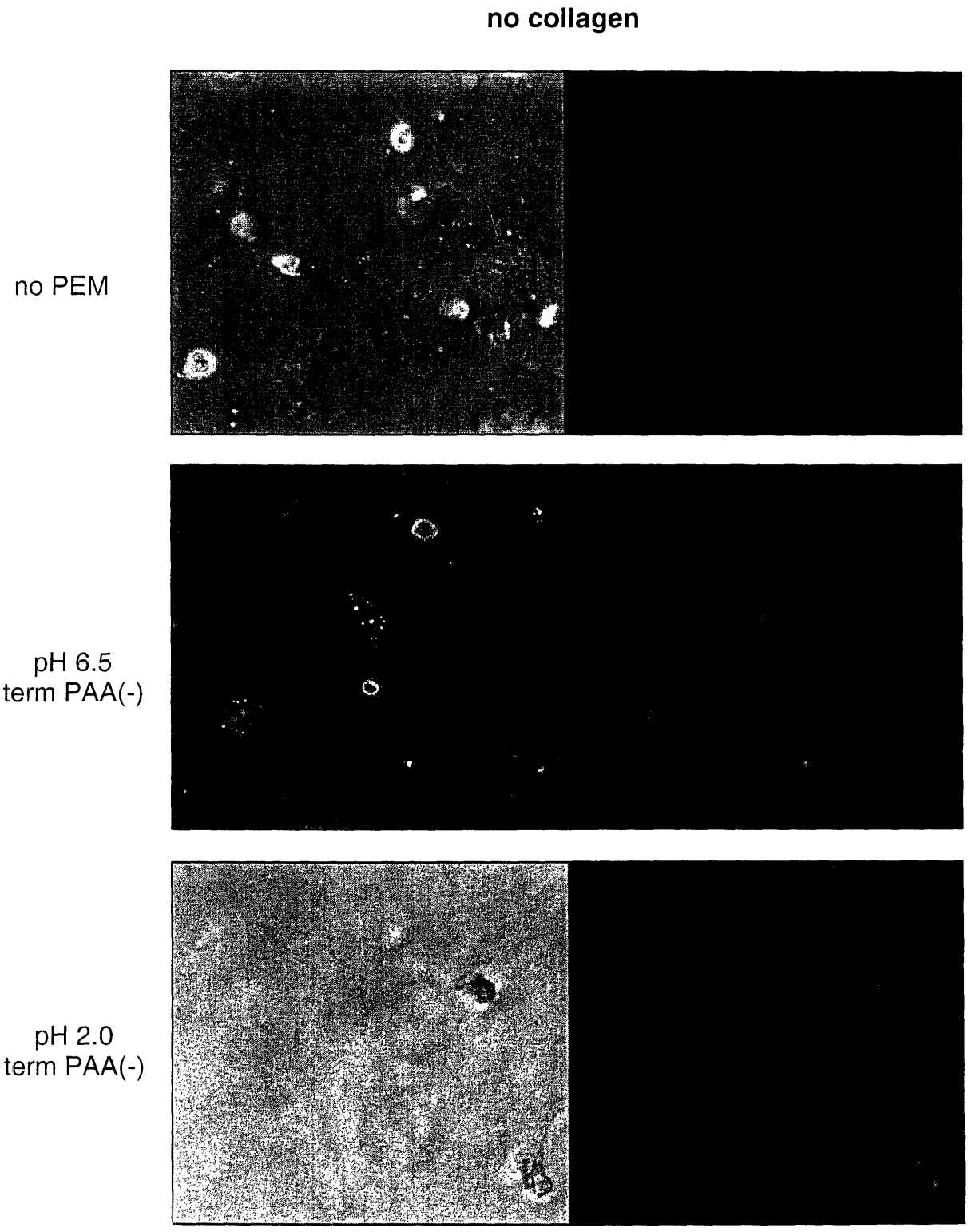
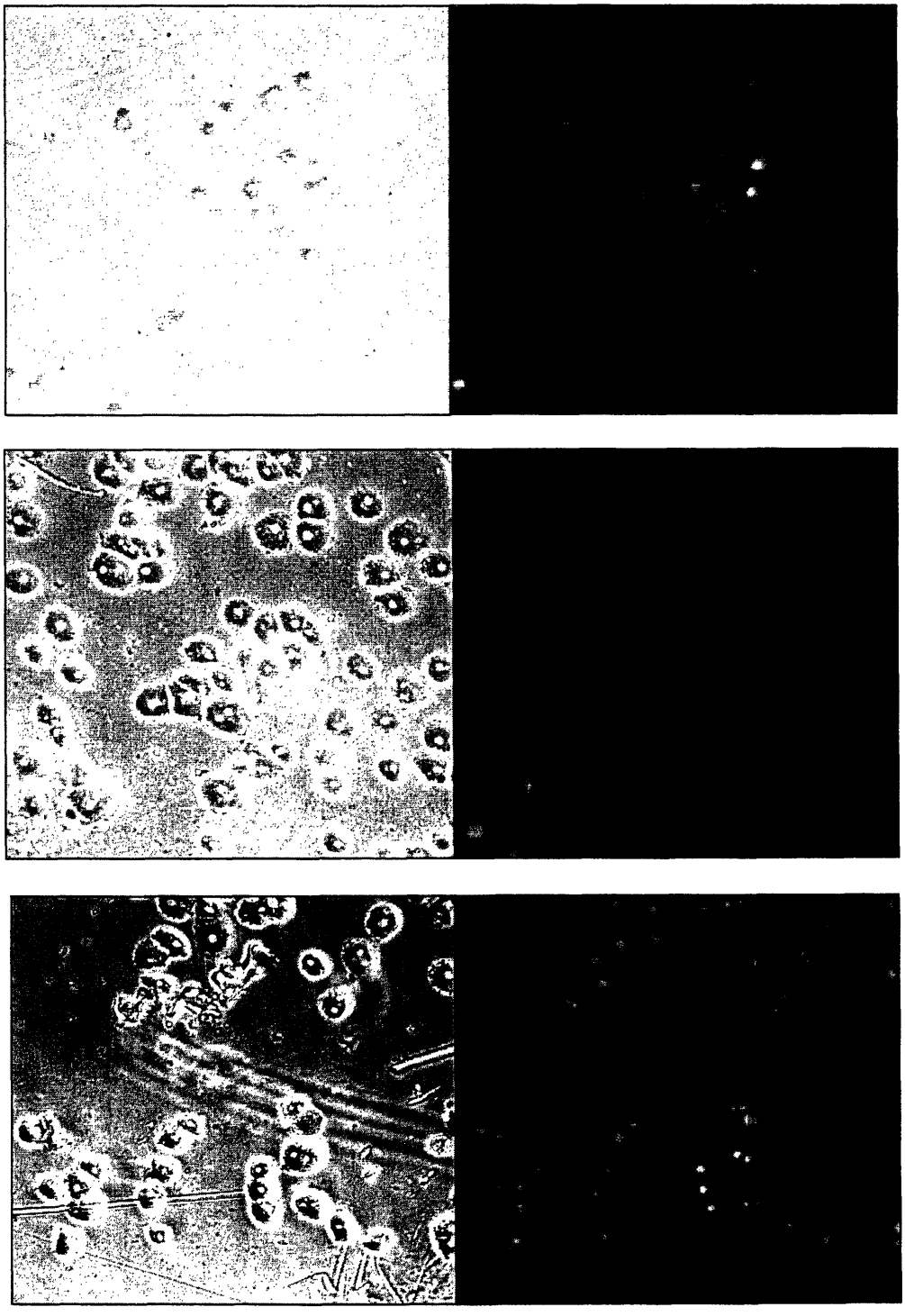


Figure 9G

Permanox

+ 10μg/cm^2 collagen



culture duration: **4 days**

VI. Future Directions and Conclusion

There are still several steps we would have liked to have done had there been more time left in the semester. Unfortunately, the experiments were extremely time consuming for a number of reasons including the amount of time it took to manufacture the circular substrates, the time it took to assemble PEM films (anywhere from 8 hours to 36 hours to deposit depending on the pH condition), reserving vacant times for the highly demanded automated dipping machines in the Rubner Lab, and, unavoidably, the cell culture duration. Regardless, the next step in this exploration will be to experimentally confirm the Young's modulus values using nanoindentation across the surfaces and PEM modifications. Secondly, hepatocyte functional characterization beyond attachment will be explored. For instance, the change in differentiated states on the surfaces which could be monitored by the secretion of albumin, a functional marker of differentiation, or by the gene transcriptional level of hepatocyte enriched transcriptional factors (i.e., HNF1 and HNF4) by RT-PCR (Sivaraman et al. 2005). Lastly, we will apply PEM modifications to polymeric scaffolds in three-dimensional diffusive bioreactors. Just as we have done for the two-dimensional cultures, we will apply and optimize conditions in three-dimensional cultures for cell attachment, morphology, and differentiated functionality on the order of a week by again monitoring through albumin secretion and transcriptional profiling.

It is unquestionable that the use of polyelectrolyte multilayers can be used to optimize cellular attachment and function to create a more natural hepatic morphology. The tunable mechanical compliance creates a manageable system that can be used to manipulate a surface to accommodate any cell type, not just hepatocytes. It is apparent that hepatocytes are determined by its biomechanical environment and, as a result, respond physically in its morphology;

likewise, it is also evident from our experiments that these cells are reliant on their biochemical surroundings, as seen by their cellular attachment frequency in the presence of collagen. Thus, in conjunction with proper collagen coating, PEM deposition can be an effective method in facilitating a healthier and more natural phenotype, resulting in organ-equivalent levels of tissue function that better resemble the liver. Though a higher amount of collagen is required to effectively coat the PEM-modified substrates, it is a small price to pay for the many advantages that these ionically cross-linked films can provide. As we have observed, PEM films can effectively mask any inherent substrate material properties. Thus, with the use of PEM modifications the variety of surfaces and geometries for hepatocyte cultures is limitless. Nevertheless, it is undeniable that the uses of polyelectrolyte multilayers has already proven to be an invaluable tool in optimizing cellular attachment and function in these hepatocyte culture experiments and will prove to be essential to future *in vitro* hepatocyte studies.

VII. Acknowledgement

The author would like to thank Jim Serdy for his help in substrate manufacturing and Jenny Lichter and the Rubner Lab for their expertise and equipment for PEM deposition. The author would like to especially thank Professor Linda Griffith for the wonderful opportunity to work on this project and Ben Cosgrove for all of his unconditional help and guidance throughout the duration of the project.

VIII. References

Davis GE, Bayless KJ, Mavila A. 2002. Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. Anat Rec 268:252-275.

Hansen LK, Hsiao C, Friend JR, Wu FJ, Bridge GA, Remmel RP, Cerra FB, Hu W. 1998. Enhanced morphology and function in hepatocyte spheroids: A model of tissue self-assembly. Tissue Eng 4:65-74.

Huang S, Ingber DE. 2000. Shape-dependent control of cell growth, differentiation, and apoptosis: switching between attractors in cell regulatory networks. Exp Cell Res 261:91-103.

Huang S, Chen CS, Ingber Del. 1998. Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. Mol Biol Cell 9:3179-3193.

Li AP, Roque MA, Beck DJ, Kaminski DL. 1992. Isolation and culturing of hepatocytes from human livers. J Tiss Cult Meth 14:139.

Powers MJ, Janigian DM, Wack KE, Baker CS, Stolz DB, Griffith LG. 2002. Functional Behavior of Primary Rat Liver Cells in a Three-Dimensional Perfused Microarray Bioreactor. Tissue Eng 8.

Semler EJ, Lancin PA, Dasgupta A, Moghe PV. 2004. Engineering Hepatocellular Morphogenesis and Function via Ligand-Presenting Hydrogels With Graded Mechanical Compliance. Biotechnol Bioeng 89:296-307.

Semler EJ, Moghe PV. 2001. Engineering hepatocyte functional fate through growth factor dynamics: the role of cell morphologic priming. Biotechnol Bioeng 75:510-520.

Sidhu JS, Farin F, Omiecinski CJ. 1993. Influence of extracellular matrix (ECM) overlay on phenobarbital medicated induction of CYP 2B1, 2B2, and 3A1 genes in primary adult rat hepatocyte culture. Biochem Biophys 301:103-111.

Singhvi R, Kumar A, Lopez GP, Stephanopoulos GN, Wang DIC, Whitesides GM, Ingber DE. 1994a. Engineering cell shape and function. Science 264:696-698.

Singhvi R, Stephanopoulos G, Wang DIC. 1994b. Review: effects of substratum morphology on cell physiology. Biotechnol Bioeng 43:764-771.

Sivaraman A, Leach JK, Townsend S, Iida T, Hogan BJ, Stolz DB, Fry R, Samson LD, Tannenbaum SR, Griffith LG. 2005. A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. Curr Drug Metab 6:569-91.

Thompson MT, Berg MC, Tobias IS, Rubner MF, Van Vliet KJ. 2005. Tuning Compliance of Nanoscale Polyelectrolyte Multilayers to Modulate Cell Adhesion. Biomaterials 26:6836-6845.

Torok E, Pollok JM, Ma PX, Kaufmann PM, Dandri M, Petersen J, Burda MR, KluthD, Perner F, Rogiers X. 2001. Optimization of hepatocyte spheroid formation for hepatic tissue engineering on three-dimensional biodegradable polymer within a flow bioreactor prior to implantation. Cells Tissues Organs 169:34-41.

Yuasa C, Tomita Y, Shono M, Ishimura K, Ichihara A. 1993. Importance of cell aggregation for expression of liver functions and regeneration demonstrated with primary cultured hepatocytes. J Cell Physiol 156:522-530.

IX. Appendix: Hepatocyte Growth Medium

HGM (Hepatocyte Growth Medium)

Block et al., J. Cell Biol. (1996), 132(6): 1133-1149 (with corrections of typos in paper)

Base Medium:

DMEM, low glucose, pyridoxine HCl, sodium pyruvate, no glutamine, no phenol red; (500ml) — GIBCO 11054-020

Add to Base Medium:

1. **0.015g L-Proline**; 0.03g/l in medium; SIGMA P-4655
2. **0.050g L-Ornithine**; 0.10g/l in medium; SIGMA O-6503
3. **0.1525g Niacinamide**; 0.305g/l in medium; SIGMA N-0636
4. **0.500g D-(+)-Glucose**; 2.25g/l in medium (already has 1g/l); SIGMA G-7021
5. **1.000g D-(+)-Galactose**; 2g/l in medium; SIGMA G-5388
6. **1.000g Bovine Serum Albumin**, Fraction V; 2g/l in medium; SIGMA A-9647
7. **Trace Metals** : Add 5µl from stock solutions: Stock concentrations:
   5.44 mg/ml $ZnCl_2$
   7.50mg/ml $ZnSO_4 7H_2O$
   2.0mg/ml $CuSO_4 5H_2O$
   2.5mg/ml $MnSO_4$
   (Use 1 Liter of MilliQ water to prepare each stock solution)

Filter the above solution with Nalgene PES Filter Unit

Add to Sterile Filtered Medium:

8. **5ml Penicillin/Streptomycin** (sterile); SIGMA P-0781
   dispense stock into 5.5ml aliquots, store at –20°C.
9. **2.5 ml L-Glutamine** (sterile); 1mM in medium; GIBCO 25030-081 (100ml); dispense 200mM stock into 13.0 ml aliquots, store at –20°C.
10. **500µl Insulin-Transferrin-Sodium Selenite** (sterile); ROCHE 1074 547
    5mg/l-5mg/l-5µg/l in medium; ROCHE 1074 547 (50mg); 1213 849 (250mg); dissolve 50mg or 250mg powder in 5ml or 25ml sterile milliQ water, dispense into 520µl aliquots, store at –20°C.
11. **400µl Dexamethasone** (sterile); 0.1µM in medium; SIGMA D-8893
    dissolve 1mg in 1ml EtOH using sterile syringe and needle, after powder is dissolved add 19ml PBS, mix thoroughly, dispense into 420µl aliquots, store at –20°C, expires 3 months from date of reconstitution.

Add to Medium Immediately Prior to First Use:

12. **200µl Epidermal Growth Factor (EGF)** (sterile); BD Biosciences 354001
    20ng/ml in medium; dissolve 100ug powder in 2 ml sterile milliQ water, dispense into 220µl aliquots, store at –20°C, expires 3 months from date of reconstitution.